Antigenic Detection of *Mycobacterium bovis* in Fresh and Fermented Milk Sold in Gombe Metropolis, Nigeria

Abstract

Milk production has increase in most developing countries and *M. bovis* has been in fresh raw and fermented nono in various parts of Nigeria. This study was carried out to detect *Mycobacterium bovis* in fresh and fermented milk sold in nono markets in Gombe Metropolis of Gombe state Nigeria. 180 samples comprise of 90fresh and 90 fermented milk samples were collected from the three (3) different nono markets. Thirty (30) samples each of fresh and fermented milk were collected from the three nono markets within Gombe metropolis. The samples were cultured on Lowenstein – Jensen Pyruvate media, and the isolates were identified using acid-fast staining, biochemical test and SD-bioline.

Twenty-four 24(26.67%) samples of both fresh and fermented milk showed a positive growth on LJ media, all the isolates of the fresh milk were positive when subjected to Ziehl-Neelsen but only seven 7(7.78%) isolates from fermented milk samples were positive after Ziehl-Neelsen staining. However, 21(23.33) and 6(6.67%) isolates were positive by SD-bioline antigenic determination test for fresh and fermented milk samples. A total of 27(15.0%) isolates were isolated from 180 samples of both fresh and fermented milk. Presence of *Mycobacterium bovis* in fresh and fermented milk and other milk products poses a health hazard as it causes extra-pulmonary tuberculosis.

Keywords: Detection, *Mycobacterium bovis*, Milk, Gombe, Metropolis.

Introduction

Bovine tuberculosis is a generally chronic respiratory disease, which is clinically difficult to diagnose although emaciation, loss of appetite, chronic cough and other signs of pneumonia could be symptoms developing at relatively late stages of the infection in cattle (Ayele et al., 2004). Bovine tuberculosis pathology is characterized by the formation of granulomatous lesions, which can within the course of the disease progress or exhibit extensive necrosis, calcify or liquefy and subsequently lead to cavity formations (Cassidy, 2006; Hope and Villarreal-Ramos, 2008). However, the bacteria can also develop a systemic infection, disseminate within its host and affect other organs (Coetzer and Tustin, 2004).

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As all *Mycobacterium* spp., *M. bovis* has an unusual cell wall surface structure characterized by the dominant presence of mycolic acids and a wide array of lipids (Glickman et al., 2001). This waxy lipid envelope confers an extreme hydrophobicity, which renders the bacteria acid- and alcohol-fast, a feature that can be exploited to identify mycobacteria via the Ziehl-Neelsen staining technique (Steingart et al., 2003).

M. bovis can be identified on the basis of specific biochemical and metabolic properties. E.g., *M. bovis* requires pyruvate as a growth supplement, is negative for niacin accumulation and nitrate reduction, shows microaerophilic growth on Lebek medium and is generally resistant to pyrazinamide. In contrast, *M. tuberculosis* does not require pyruvate as a growth supplement, is positive for niacin accumulation and nitrate reduction, shows aerophilic growth on Lebek medium, and is usually not mono-resistant to pyrazinamide (Kubica et al., 2006; Cole, 2002). However, the unequivocal validity of these characteristics is challenged by several studies (Kubica et al., 2006; Niemann et al., 2000). Different molecular markers and techniques have been discovered and developed in the past that allow the unambiguous identification and differentiation of *Mycobacterium* spp. and the members of the MTBCS (Huard et al., 2006; Pinsky and Banaei, 2008). This study was design to detect the present of *Mycobacterium bovis* using culture, microscopy and SD-bioline antigenic determination test in fresh and fermented milk sold in Gombe metropolis. The study was designed to detect the presence of *Mycobacterium bovis* in both fresh and fermented milk sold in the three major nono markets (Gombe main market, Tashan Shongo market and Tashan Dukku market) within Gombe metropolis.

Materials and Methods

Sample Collection

A total of one hundred and eighty (180) samples were collected for this study. Ninety (90) samples each of fresh and fermented milk samples were collected and thirty (30) samples of both fresh and fermented milk samples were collected from each of the nono market within Gombe metropolis. The samples were collected in sterile corked plastic bottles and transported to the laboratory in an iced container.

Detection of *Mycobacterium bovis* in Milk Samples

Preparation of Lowenstein – Jensen medium

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Lowenstein-Jensen media was prepared according to the manufacturer's instruction. 37.2g of the Lowenstein – Jensen media was weighed and dissolved in 600mL of distilled water. The media was swirled very well to dissolve the powder; after which 7.2g of sodium pyruvate was measured and added to the medium with continuous shaking until the medium became completely homogenized. The medium was then sterilized by autoclaving at 121^0C for 15minutes and then removed and allowed to cool.

Fresh eggs were then carefully cleansed with soap and water before they were placed in 70% ethanol for 15minutes. The eggs were then removed and allowed to dry and then emptied inside a sterile blender and blended. It was then allowed to settle before filtering through sterile cotton gauze. The filtered egg was gently mixed with the media to homogeneity. The medium was then dispensed in 6 - 8mL volumes into sterile McCartney bottles, the bottles were placed on the racks to achieve appropriate slope and they were inspissates at 80^0C for 45minutes. The bottles were cooled and labeled to identify the batch and the date of preparation. The bottles were then kept in an upright position in the refrigerator (WHO, 1998).

Procedure for Decontamination

Fifteen ml (15mL) of the fermented milk sample was measured and put into a falcon tubes and equal amount of 4% NaOH was added to the sample. The falcon tubes containing the samples were then closed tightly and shaken to digest the sample and then allowed to stand for 15minutes at room temperature with occasional shaking. This was then centrifuged at 3000rpm for 15 minutes. After centrifugation, the supernatant was removed; 15mL of sterile saline was then added to the sediment in the falcon tubes and then centrifuged further for another 15minutes at 3000 rpm. Thereafter, the supernatant was decanted and then the sediment was inoculated onto a Lowenstein – Jensen culture media (WHO, 1998).

Isolation of *Mycobacterium bovis*

For the isolation of *Mycobacterium bovis*, a method reported by of Ben *et al.,* was adopted. The samples were decontaminated using 4% NaOH, and then neutralized with sterile normal saline, centrifuged at 3000rpm for 30minutes. The deposits after centrifugation and decantation of supernatant were inoculated onto the already prepared Lowenstein – Jensen medium and incubated at 37^0C for 7 - 8 weeks (Ben Kahla et al., 2011).

Identification of *Mycobacterium bovis*

Ziehl–Neelsen Staining Procedure

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A smear of colonies from growth on LJ medium was prepared allowed to dry and then heat fixed. The smear was then flooded with carbol fuchsin stain and heated for 5 minutes and then rinsed with de- ionized water. Acid alcohol was then flooded on the smear for 15 seconds until the smear is sufficiently decolorized i.e. pale pink. The slide was then rinsed with de - ionized water and Loffler's Methylene Blue stain was added as counter stain for 60 seconds. After which it was rinsed with clean water and allowed to dry. The smear was then examined microscopically using x10 oil immersion (Delisle and Tomalty, 2002; Cheesbrough, 2006).

Biochemical Analysis

The colonies were subjected to certain biochemical test such as niacin accumulation test and nitrate reduction test as described by Palomino *et al.,* to differentiate *M. bovis* from *M. tuberculosis*. Isolates that were niacin and nitrate negative were identified as *Mycobacterium bovis* (Cheesbrough, 2006).

Nitrate Reduction Test

The isolates were into a buffer solution containing nitrate and incubated at 37^0C for 2hours. Then sulphanilamide and n-naphthylenediamine dihydrochloride solution was added. Positive reaction was indicated by the presence of pink to red color within 30 – 60 seconds (Arora and Arora, 2007).

Niacin Accumulation Test

Three to four (3 – 4) culture slant of Lowenstein-Jensen slant was flooded with 1 ml of distilled water. The medium was stabbed with the tip of the pipette to allow access of the water to the underlying medium. The tube was tilted to allow the water to covers the surface of the slant. The tube was left to stand for 20 to 30 minutes. The tube was rotated so that the slant faces downward. Carefully, 0.6 ml of extract was transferred to the screw cap test tube and then added 0.25 ml of o-toludine and 0.25 ml of 10% cyanogen bromide. The tube was observed formation of a pink color which indicate positive test within 5 minutes (Cheesbrough, 2006).

SD-Bioline Test for the Confirmation of *Mycobacterium bovis*

SD–Bioline Rapid test was performed according to the manufacturer's guidelines. Three to four colonies of mycobacterial strains grown in Löwenstein-Jensen media were emulsified in 100µL of extraction buffer, and then 50µL of the extraction buffer was placed in sample well on the test strip. The results were visually assessed based on color development after incubation at room

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temperature for 15 min. The presence of two-color bands in the control and test window was regarded as positive result while presence of only control band indicates a negative result (WHO, 2006; Hyeon-Seop et al., 2015).

Statistical Analysis

The Pearson Chi-square test was used to determine significance of results and p. value < 0.05 was considered statistically significant.

Results

M. bovis was grown and isolated from fresh and fermented milk on Lowenstein-Jensen media. The result of the study revealed that out of the samples collected from Gombe main market, ten (10) fresh and eight (8) fermented milk samples were positive on LJ media which is equivalent to 33.33% and 26.67% respectively. However, from the samples collected from Tashan Dukku market, seven (7) fresh and nine (9) fermented milk samples were positive on LJ media with a frequency of 23.33% and 30.00% respectively.7(23.33%) samples from fresh and fermented collected from Tashan Shongo market were positive on LJ media. Furthermore, a total of 24(26.67%) samples were found to be positive on LJ media from both fresh and fermented milk as described in Table 1.

Table 1: Result of Isolation of *M. bovis* from Fresh and Fermented Milk Samples on Lowenstein- Jensen Medium

		Fresh		Fermented	
Locations	**No of Samples**	**Growth on LJ Media**	**% of Positive on LJ Media**	**Growth on LJ Media**	**% of Positive on LJ Media**
GMM	30	10	33.33	8	26.67
TDM	30	7	23.33	9	30.00
TSM	30	7	23.33	7	23.33
Total	90	24	26.67	24	26.67
		p value = 0.749		p value = 0.992	

Key: LJ = Lowenstein Jensen , Gombe Main Market (GMM) , Tashan Dukku Market (TDM), Tashan Shongo Market (TSM).

Table 2 described the result of the Ziehl-Neelsen staining of the isolates. All the isolate obtained from fresh milk samples were ZN positive, but only 3 of the 8 isolates obtain from fermented

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milk in GMM are ZN positive; and 2of the 9 and 7 isolates obtained from the fermented milk in TDM and TSM respectively were found to be positive by Ziehl-Neelsen staining. Hence, a total of twenty-four (24) and seven (7) isolates from fresh and fermented milk respectively were found to be positive by Ziehl-Neelsen staining procedure.

Table 2: The Result of Ziehl-Neelsen Staining

		Fresh Milk		Fermented Milk	
Locations	**No of Samples**	**No of Positive by ZN**	**% of positive by ZN**	**No of Positive by ZN**	**% of positive by ZN**
GMM	30	10	33.33	3	10.0
TDM	30	7	23.33	2	6.67
TSM	30	7	23.33	2	6.67
Total	90	24	26.67	7	7.78
		p value = 0.993		p value = 0.993	

Key: ZN = Ziehl-Neelsen, Gombe Main Market (GMM), Tashan Dukku Market (TDM), Tashan Shongo Market (TSM).

Table 3 depicted the result of SD-bioline after Ziehl-Neelsen staining. The isolates that showed positive reaction after the Ziehl-Neelsen staining were subjected to SD-bioline test to further identify the organisms. The result indicated that all the ZN positive isolates obtained in fresh milk samples from TDM are SD-bioline positive but 2 out of the 10 isolates obtained from GMM fresh milk samples are SD- bioline negative. Also 6 out the 7 isolates obtained from TSM fresh are SD-bioline positive. However, all the isolates obtained in fermented milk from Tashan Dukku and Tashan Shango markets that are ZN positive were found to be SD-bioline positive while one (1) isolate from those obtain in samples collected from Gombe main market is SD-bioline negative and the remaining 2 isolates were positive.

Table 3: The Result of SD- Bioline Antigenic Test

		Fresh Milk		Fermented Milk	
Locations	**No of Samples**	**No of SD-Bioline Positive**	**% of SD-Bioline Positive**	**No of SD-Bioline Positive**	**% of SD-Bioline Positive**
GMM	30	8	26.67	2	6.66
TDM	30	7	23.33	2	6.66
TSM	30	6	20.00	2	6.66
Total	90	21	23.33	6	6.66
		p value = 0.978		p value = 0.999	

Key: Gombe Main Market (GMM), Tashan Dukku Market (TDM), Tashan Shongo Market (TSM)

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Discussion

Study on the incidence of *Mycobacterium bovis* in fresh and fermented milk revealed the presence of the organisms in the samples analysed. The study also depicted that all the samples collected from different study areas (Gombe main market, Tashan Dukku market and Tashan Shongo markets) were found to be contaminated with *Mycobacterium bovis*. However, higher prevalence of *Mycobacterium bovis* was found in fresh milk samples than fermented milk. From the present study, 21(23.33%) of the fresh milk samples collected and analysed were found to be contaminated with *M. bovis* while only 6(6.67%) samples out of 90 fermented samples collected analysed in the study were found to be contaminated with *M. bovis*. However, the results of this study for fermented milk samples 6(6.66%) is lower than the those reported by Ogundeji *et al.* (2015) who detected 8(16%) of positive milk samples for *M. bovis* in their study conducted on Molecular detection of *Mycobacterium bovis* in cattle milk in Enugu State, Nigeria. The result is also higher in fresh milk than those reported by Ogundeji et al. (2015).

A study conducted by Ofukwu et al. (2008) reported the presence of *Mycobacterium bovis* 2(2.2%) of 90 "nono" samples analyzed in Makurdi, Benue State of Nigeria which is lower than the result obtained in this study for both fresh and fermented milk. In another study, Abubakar, (2007) reported prevalence rate of 14(12.6%) of *Mycobacterium bovis* from 111 cow milk samples in Federal capital territory and Kaduna State of Nigeria; this result is higher than the result of the present study in fermented milk and lower than those obtain in fresh milk.

However, 6(6.67%) reported in this study in fermented milk is in line with the result reported by Cadmus et al. (2004) who detected 6(11.3%) *Mycobacterium bovis* from 53 milk samples screened in Ibadan, Oyo state of Nigeria. The result in fermented milk is also significantly lower than findings of Ofukwu *et al.*, who reported 4(18.2%) of *Mycobacterium bovis* from 22 cow milk in Makurdi, Nigeria.

Milk contamination by microorganisms generally occurs from three main sources; from within the udder, from the exteriors of the udder and also from the surfaces of milk handling and storage equipment's (Bramley and Mckinnon, 1990). Moreover, Murphy and Boor, (2000), reported that the health and hygiene of the cow, the environment in which the cow is housed and milked, and the procedures used in cleaning and sanitizing the milking and storage equipment, all influence

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microbial numbers in milk. Temperature and length of storage time are important because they can support the growth of microorganisms in milk (Murphy and Boor, 2000).

Large number of the inhabitants of the study area uses milk as a source of food and they usually buy the milk from milk vendors who are running from one street to another and sometimes they go to nono market to buy. Hence, detection of *Mycobacterium bovis* from cow milk poses a serious threat to the individuals that consumed the milk. This is because when cow milk is produced in small quantity it is not always sold to dairy industries for pasteurization, rather sold at retail and may be consumed raw and it also be used for the production of fermented dairy products.

Conclusion

This study detects the presence *Mycobacterium bovis* in both fresh and fermented cow milk sold in the nono markets within Gombe metropolis. The study revealed fermented milk is safer for consumption than fresh milk since it has a lower prevalence of *M. bovis*. The results also reveal that there is chance of transmitting *Mycobacterium bovis* from animals to human through consumption of poorly treated and unhygienic cow milk. The occurrence of zoonotic diseases such as those causes by *Mycobacterium* species in milk could be acquired by humans through consumption of milk contaminated by these organisms leading to pulmonary and extra-pulmonary tuberculosis. However, cow milk gets easily contaminated during milking if the udder is not properly clean. *Mycobacterium bovis* can be transferred from the udder of the cow into the container during milking which if the milk is not properly pasteurized it can lead to zoonotic infection.

Recommendation

Clinicians and veterinarians should be aware of the occurrence of *Mycobacterium bovis* in the stable diet of residents (Milk) in the study area for proper diagnosis and treatment as the case may be. Also, accurate measured should be taken during milking and also during processing of the milk to form fermented milk. Public health awareness should be conducted especially to the peoples milking and processing the milk as well as those vendors selling the cow milk.

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